

# In Vitro Sensitivity of *Plasmodium falciparum* from China-Myanmar Border Area to Major ACT Drugs and Polymorphisms in Potential Target Genes

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## Abstract

Drug resistance has always been one of the most important impediments to global malaria control. Artemisinin resistance has recently been confirmed in the Greater Mekong Subregion (GMS) and efforts for surveillance and containment are intensified. To determine potential mechanisms of artemisinin resistance and monitor the emergence and spread of resistance in other regions of the GMS, we investigated the *in vitro* sensitivity of 51 culture-adapted parasite isolates from the China-Myanmar border area to four drugs. The 50% inhibitory concentrations (IC<sub>50</sub>) of dihydroartemisinin, mefloquine and lumefantrine were clustered in a relatively narrow, 3- to 6-fold range, whereas the IC<sub>50</sub> range of artesunate was 12-fold. We assessed the polymorphisms of candidate resistance genes *pfcr*, *pfmdr1*, *pfATP6*, *pfmdr6* and *pfMT* (a putative metabolite/drug transporter). The K76T mutation in *pfcr* reached fixation in the study parasite population, whereas point mutations in *pfmdr1* and *pfATP6* had low levels of prevalence. In addition, *pfmdr1* gene amplification was not detected. None of the mutations in *pfmdr1* and *pfATP6* was associated significantly with *in vitro* sensitivity to artemisinin derivatives. The ABC transporter gene *pfmdr6* harbored two point mutations, two indels, and number variations in three simple repeats. Only the length variation in a microsatellite repeat appeared associated with altered sensitivity to dihydroartemisinin. The *PfMT* gene had two point mutations and one codon deletion; the I30N and N496—both reached high levels of prevalence. However, none of the SNPs or haplotypes in *PfMT* were correlated significantly with resistance to the four tested drugs. Compared with other parasite populations from the GMS, our studies revealed drastically different genotype and drug sensitivity profiles in parasites from the China-Myanmar border area, where artemisinins have been deployed extensively for over 30 years.

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## Introduction

The development and spread of multidrug resistant (MDR) *Plasmodium falciparum* has led to the adoption of artemisinin-based combination therapies (ACTs) as the first-line treatment for falciparum malaria in most malaria-endemic countries of the world [1]. Artemisinin and its derivatives are by far the most potent antimalarial drugs [2], and at present, our last line of defense against multidrug resistant parasites. Therefore, the recently confirmed emergence of artemisinin resistance in western Cambodia is a major threat for current initiatives to control and eliminate malaria [3–5]. Because this exact same area has been the origin of both chloroquine (CQ) and sulfadoxine-pyrimethamine resistance, both of which have subsequently spread to Africa [6,7], the consequence of a similar spread of artemisinin resistance will be catastrophic. While limited evidence suggests that artemisinin

resistance has not yet spread to other areas [8], the World Health Organization (WHO) is coordinating a large-scale elimination campaign in this region aiming to contain artemisinin resistance [9,10]. Apparently, containment efforts require better resistance surveillance [11], but this is hampered due to the lack of convenient molecular surveillance tools for detecting artemisinin resistance. At this moment, the most reliable way of detecting artemisinin resistance is through rigorous clinical efficacy studies, which are expensive and time-consuming.

The mode of action of artemisinins in malaria parasites is still a debated topic and the molecular basis of reduced artemisinin susceptibility is unclear [12–14]. To date, a few genes have been postulated to be associated with artemisinin resistance. The *P. falciparum* multiple drug resistance 1 (*pfmdr1*) gene has received the most attention, because several mutations (N86Y, Y184F, S1034C,

N1042D, and D1246Y) occurring in PfMDR1 from field isolates are associated with altered sensitivity to multiple structurally unrelated antimalarials such as CQ, mefloquine (MQ), quinine (QN), halofantrine (HF), and artemisinins [15–19]. In addition, *pfmdr1* amplification is a key determinant for both *in vivo* and *in vitro* resistance to MQ and HF [19–23]. Increased *pfmdr1* copy number, which is more prevalent in west Cambodia, is associated with increased risk of therapy failures of artesunate (AS)-MQ, the major ACT deployed in Thailand and Cambodia [24–28]. The sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA) homologue PfATP6 has been considered as a specific target of artemisinins, since artemisinin derivatives inhibit this enzyme expressed in *Xenopus* oocytes [29] and this inhibition was abolished by the introduction of the L263E mutation in the predicted transmembrane domain 3 of PfATP6 [30]. Another mutation (S769N) has been linked to artemether resistance in *P. falciparum* field isolates from French Guiana [31]. However, the L263E mutation has not been found in field isolates from most malaria endemic areas, and S769N is very rare [32–40]. Though multiple new single nucleotide polymorphisms (SNPs) have been detected in *pfatp6*, their associations with artemisinin resistance have not been established. Mutations in several other genes also have been suggested to be responsible for artemisinin resistance. Mutations in the *UBP1* gene encoding a deubiquitination enzyme have been identified to confer artemisinin resistance in the rodent malaria parasite *P. chabaudi* [41]. However, the equivalent mutations have not been found in *P. falciparum* field isolates from Cambodia and Thailand [42]. Recently, using a genome-wide association approach, Mu et al. detected signs of positive selection at several putative transporter genes in parasite populations including one coding the ABC transporter *pfmdr6* and another gene coding the metabolite/drug transporter *pfMT* [43,44]. So far, none of the candidate genes have been conclusively shown to be responsible for artemisinin resistance. Therefore, further research is needed to identify the causal mutations for artemisinin resistance.

Malaria is still a serious public health problem in the Greater Mekong Subregion (GMS), which includes Cambodia, Laos, Thailand, Vietnam, Myanmar and China [45]. Within this region, malaria transmission is particularly intense along international border areas. The GMS is historically a hotspot of antimalarial drug resistance, and resistance management remains a high priority for malaria control. The China-Myanmar border area has the longest history of artemisinin monotherapy; it has been used for over three decades and *in vitro* studies have detected reduced susceptibility to artemisinins [46]. Therefore, close surveillance of artemisinin resistance in this area is necessary to detect and deter resistance development. Here we report an assessment of *in vitro* sensitivity of clinical *P. falciparum* isolates from the China-Myanmar border area to four antimalarial drugs, AS, dihydroartemisinin (DHA), lumefantrine (LMF) and MQ. Using a candidate gene approach, we want to determine whether polymorphisms in these genes are associated with decreased drug sensitivity.

## Materials and Methods

### Parasite Samples and *in vitro* Culture

*P. falciparum* clinical isolates were collected in 2007–2009 from symptomatic patients presenting with uncomplicated *P. falciparum* infections attending a malaria clinic in Laiza township near the China-Myanmar border. Malaria infections were diagnosed by microscopic examination of Giemsa-stained thick and thin blood films. To confirm monoclonal infections, parasites were genotyped at three polymorphic genes, *merozoite surface protein 1 (msp1)*, *msp2* and *glutamate-rich protein (glurp)*, using previously described methods

[47–49]. A total of 21, 9, and 21 parasite isolates were adapted to continuous *in vitro* culture for the year 2007, 2008, and 2009, respectively [50]. The human subject protocol for this study was approved by the Institutional Review Board of Kunming Medical University. Parasite routine culture was maintained in type O<sup>+</sup> human red blood cells (RBCs) in complete medium supplemented with 6% AB human serum under an atmosphere of 90% N<sub>2</sub>/5% O<sub>2</sub>/5% CO<sub>2</sub> [51].

### *In vitro* Drug Sensitivity Assay

A SYBR Green I-based fluorescence assay was used to measure parasite sensitivity to four antimalarial drugs: AS, DHA, MQ, and LMF [52]. MQ was purchased from Sigma (St Louis, MO, USA), while the other three drugs were obtained from Kunming Pharmaceutical Co. (Kunming, Yunnan, China). The stock solution of AS (0.260 mM), DHA (0.352 mM), MQ (2.56 mM), and LMF (7.56 mM) were prepared in ethanol and 2-fold dilutions were made in complete medium. Cultures were synchronized by two rounds of sorbitol treatment, and late ring or early trophozoite stage parasites were assayed for drug sensitivity in 96-well microtiter plates at 5% hematocrit and 0.3% parasitemia. For drug assays, 90  $\mu$ l of parasite culture were seeded into each culture well, and 10  $\mu$ l of diluted drugs in complete medium solutions were dispensed into each well to obtain a desired final concentration. The plates were incubated at 37°C in a CO<sub>2</sub> incubator for 72 h. The plates were then frozen at –80°C, thawed, and mixed with 100  $\mu$ l of lysis buffer. The plates were incubated in the dark for about 1 h and fluorescence data were acquired using the Fluoroskan Ascent FL microplate fluorometer (Thermo Scientific, Waltham, MA). For each parasite isolate and drug concentration, the assay was performed in three biological replicates and each with three technical replicates. To reduce the variations between plates, the standard laboratory clone 3D7 was included as a reference. The *in vitro* drug response data were entered into the SPSS data editor, and the geometric mean of the half-maximal inhibitory concentration (IC<sub>50</sub>) was calculated for all isolates using a regression-probit analysis. Standard deviation was calculated using the mean values of the three biological replicates of each parasite isolate.

### Molecular Analysis of Candidate Genes

Parasite genomic DNA was isolated from cultured parasites using a proteinase K digestion and phenol/chloroform extraction procedure [53]. By using a PCR and sequencing approach, we assessed SNPs in *pfprt*, *pfmdr1*, *pfatp6*, a putative ABC transporter *PF13\_0271 (pfmdr6)*, and a putative metabolite/drug transporter *PF14\_0260 (pfMT)*. Two *pfprt* fragments covering codons 76 and 220 and two *pfmdr1* fragments including codons 86, 184, 1034, 1042 and 1246 were amplified as described previously [54]. Two regions of *pfatp6* gene (28–3065 and 3207–3952 bp) were amplified using primers and conditions from previously published work [36,55] (see Table S1). The coding sequences of *pfmdr6* and *pfMT* genes, shown to be under strong positive selection [43], were amplified using primers shown in Table S1. To minimize errors introduced in the sequences during PCR amplification, we used Advantage HD DNA Polymerase Mix (Clontech, Mountain View, CA), which has efficient 3' → 5' exonuclease activity for high fidelity. PCR products were purified using a PCR Purification kit (Qjagen, Valencia, CA) and used for direct sequencing. Overlapping sequences were obtained by using sequencing primers. For singletons, the sequence was confirmed from two independent PCR reactions from the same DNA templates. For a highly AT-rich region in the *pfMT* gene, the primers MT\_873 and mt\_1433R were used to amplify this fragment and the PCR products were

cloned into the pGEM-T Easy Vectors system (Promega, Madison, WI). Plasmid DNA was extracted by Zippy Plasmid Miniprep Kit (Zymo research, Orange, CA). For accuracy, at least two clones were sequenced for each sample. Alignment of DNA sequences were performed using the BioEdit program with the 3D7 sequence as the reference. All new sequences were deposited in GenBank under the accession numbers (JN983240–JN983290 for *pfatp6*, JN983291–JN983341 for *pfmdr1*, and JN983342–JN983392 for *pfMT*).

*Pfmdr1* copy number in field parasite isolates was determined by real-time PCR using FastStart Universal SYBR Green Master Mix on an ABI 7300 real-time PCR system (Applied Biosystems). DNA from the reference strain 3D7, which has a single copy *pfmdr1* gene, was included for calibration. The single copy  $\beta$ -*tubulin* gene served as an internal control for estimating the copy number of *pfmdr1*. Each reaction was performed in a final reaction volume of 20  $\mu$ l. Copy number of *pfmdr1* gene was calculated using the  $2^{-\Delta\Delta C_t}$  method as described earlier [56]. The efficiency of each PCR (*pfmdr1* and  $\beta$ -*tubulin*) was determined using a scale dilution of DNAs from the laboratory lines 3D7 (containing 1 copy of *pfmdr1*) and Dd2 (containing 3–4 copies of *pfmdr1*). Determination of the copy number was done by comparison of the ratio of *pfmdr1*/ $\beta$ -*tubulin* taking into account the efficiency of each PCR. Each sample was analyzed in triplicate. Assays were repeated if one of the following two results was obtained:  $C_t$  value >35, or copy number as 1.3–1.6 or <0.7.

### Statistical Analysis

Spearman’s correlation coefficients were used to investigate the degree and significance of the relation between the IC<sub>50</sub> values of the four drugs. It was chosen instead of Pearson’s in order to provide robust correlation estimates despite potential outliers in the data. We then used *t*-tests to look for significant differences in mean IC<sub>50</sub> values between these field isolates and 3D7, and between the years. Finally, we used multiple *t*-tests and a generalized linear model to investigate potential, significant differences in mean IC<sub>50</sub> values between haplotypes and point mutations for each gene and for each drug. The Bonferroni correction was used to control for the increased probability of false positives in multiple comparisons. All statistical analyses were done using SAS version 9.2 (<http://www.sas.com/>) and R version 2.14.2 (<http://cran.r-project.org/>).

## Results

### In vitro Drug Assays

To determine whether artemisinin resistance has emerged at the China-Myanmar border area, we assessed *in vitro* sensitivity of 51 culture-adapted *P. falciparum* strains originating from this region to two artemisinin derivatives AS and DHA and two aminoalcoholic drugs MQ and LMF. These parasites were collected in 2007–2009 and genotyped to confirm monoclonal infections [54]. The IC<sub>50</sub> values of individual parasite isolates are shown in Table S2. Parasite isolates were generally sensitive to AS with a mean IC<sub>50</sub> of 5.8 nM, but the range (1.4–16.5 nM) was relatively wide with an almost 12-fold difference between the most and least susceptible isolates (Table 1). The mean IC<sub>50s</sub> of the samples to DHA ranged from 9.6 to 40.3 nM with a mean IC<sub>50</sub> of 23.0 nM. The mean IC<sub>50</sub> for MQ was 50.5 nM, ranging from 16.2 to 96.2 nM, with a six-fold difference in IC<sub>50</sub> between the most and the least susceptible isolates. The parasites had a mean IC<sub>50</sub> of 5.9 nM for LMF with a fairly narrow range of 3.0–11.5 nM. Compared with the mean values of the control laboratory clone 3D7, only MQ sensitivity showed a significant difference after Bonferroni

correction ( $P < 0.05$ ). When the IC<sub>50</sub> data were stratified by years, slight variations were observed between the years (Figure 1). However, none of the comparisons were statistically significant ( $P > 0.05$ , *t*-test). Also noteworthy is the detection of several outlier isolates (greater than 2 standard deviation), which showed much higher IC<sub>50</sub> values to all drugs.

Correlations between the IC<sub>50s</sub> of the four antimalarial drugs were evaluated using the Spearman’s test (Table 2). There was a highly significant correlation between responses to the two artemisinin derivatives ( $P < 0.0001$ ). When all samples were considered, we did not detect significant correlation between the two aminoalcoholic drugs MQ and LMF ( $P > 0.05$ ). However, when only the samples collected in 2009 were compared, a modest correlation was found between MQ and LMF ( $P < 0.05$ ) (data not shown).

### Polymorphisms in Candidate Target Genes

**Pfmdr1.** We genotyped *pfmdr1* SNPs by PCR and sequencing in the 51 field isolates, focusing on the known amino acid substitutions at codons 86 (N/Y), 184 (Y/F), 1034 (S/C), 1042 (N/D), and 1246 (D/Y). Wild-type haplotype was prevalent and accounted for ~65%. Except codon 184, mutations at other codons were rare (Table 3). Specifically, mutations at codons 1034 and 1246 were not observed. The N86Y mutation, which is associated with CQ resistance and increased sensitivity to MQ and artemisinin [57], was detected only in one (2%) isolate of the samples. The N1042D mutation associated with QN resistance was also rare and found only in 4 (7.8%) isolates of the samples [18]. The C-terminal mutation haplotype 1034C/1042D/1246Y, which is prevalent in South America and enhances parasite susceptibility to MQ and artemisinin, was absent in our samples (Table 3). We did not detect *pfmdr1* amplification in any samples; the 51 isolates had a mean *pfmdr1* copy number value of  $1.02 \pm 0.14$  (Figure S1).

**Pfprt.** The key determinant of CQ resistance K76T was ubiquitously present in all samples. Further, the A220S mutation almost reached fixation and was found in 50/51 parasite isolates (data not shown).

**PfATP6.** We obtained complete sequences of the *PfATP6* coding region in 51 samples and compared them with that from 3D7. Mutations were rare and sporadic with a total of five identified (Table 3). Three mutations have been described previously (I89T, A438D and N465S), and two were novel mutations (I226V and E710K). Except for the I89T and A438D

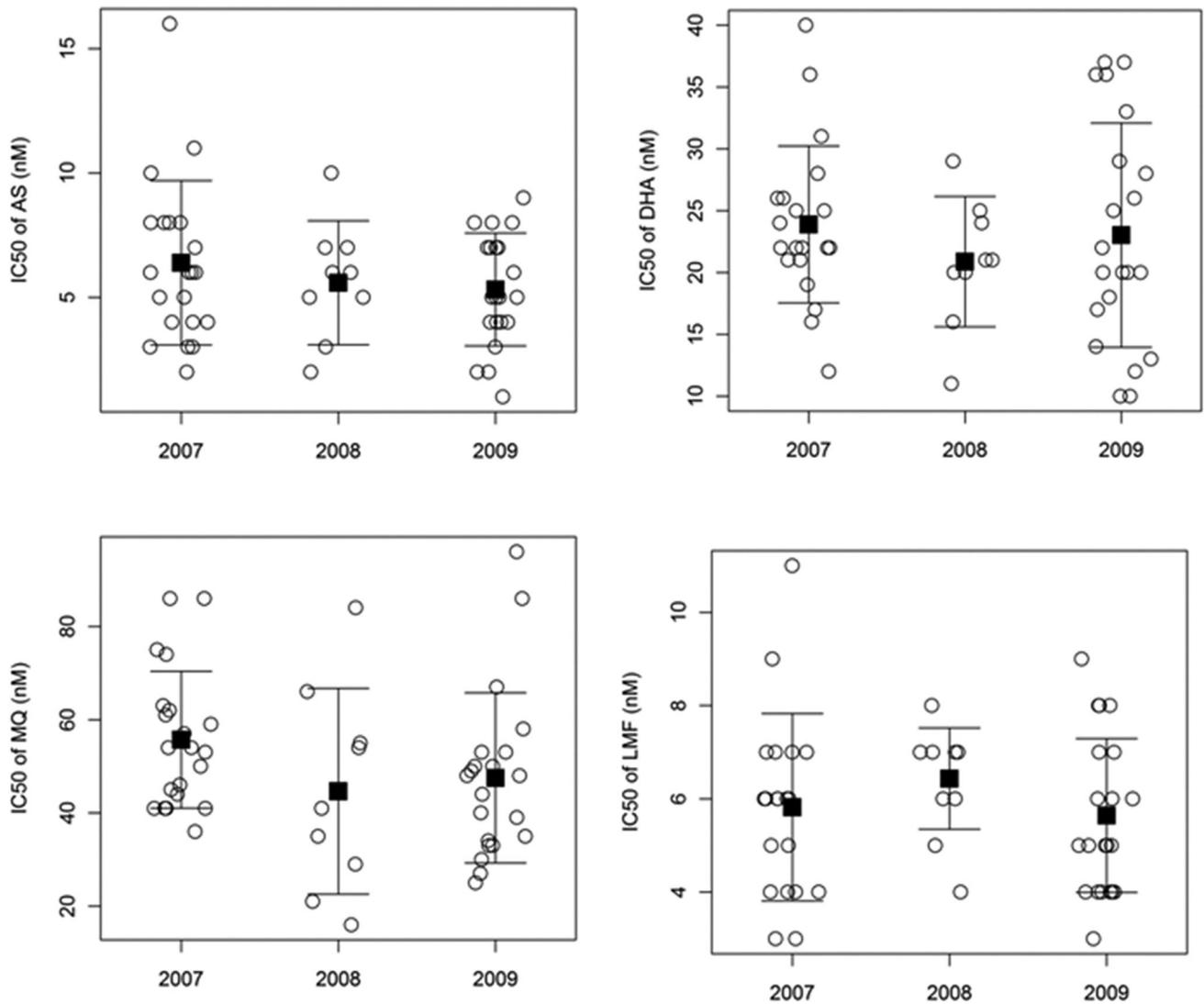
**Table 1.** *In vitro* IC<sub>50s</sub> (nM) of 3D7 and 51 culture-adapted clinical isolates to four antimalarial drugs.

Drug	3D7 (Mean $\pm$ SD)	Clinical isolates	
		Mean $\pm$ SD	Range
Artesunate	5.4 $\pm$ 1.5	5.8 $\pm$ 2.8	1.4–16.5
Dihydroartemisinin	22.1 $\pm$ 2.0	23.0 $\pm$ 7.4	9.6–40.4
Mefloquine	40.7 $\pm$ 11.7	50.4 $\pm$ 17.8*	16.2–96.2
Lumefantrine	7.12 $\pm$ 2.1	5.9 $\pm$ 1.7	3.0–11.5

IC<sub>50</sub> values are shown as mean  $\pm$  standard deviation (SD). The mean IC<sub>50</sub> of each clinical isolate was used to calculate the mean IC<sub>50</sub> and SD for all isolates. The range of IC<sub>50</sub> values of all clinical isolates is also shown for each drug. *t*-tests were used to compare the IC<sub>50</sub> values between test samples and laboratory clone 3D7. Asterisk (\*) indicates statistically significant difference from 3D7 after a Bonferroni correction ( $P < 0.0009$ ).

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**Figure 1**



**Figure 1. *In vitro* drug responses. IC<sub>50</sub>s (nM) of *P. falciparum* isolates to artesunate (AS), dihydroartemisinin (DHA), mefloquine (MQ) and lumefantrine (LMF) are stratified by year. Solid boxes (■) indicate the mean values; the error bars denote one standard deviation. For IC<sub>50</sub> values of each drug, there was no significant differences between the years ( $P > 0.05$ , *t*-test). doi:10.1371/journal.pone.0030927.g001**

mutations, which occurred in 5 (9.8%) and 3 (5.9%) isolates of the samples, respectively, the remaining mutations were only detected once in the samples. The L263E and S769N mutations, which have been proposed to confer artemisinin resistance [30,31], were

not detected in our samples. A total of seven haplotypes were detected; 43 (84.3%) were wild-type, whereas six and two samples contained single and two mutations, respectively.

**Table 2. Correlation matrix showing Spearman’s correlation coefficients between the four tested drugs.**

	Mefloquine	Lumefantrine	Dihydroartemisinin	Artesunate
Mefloquine	–			
Lumefantrine	0.2060	–		
Dihydroartemisinin	–0.0056	0.2316	–	
Artesunate	0.2523	0.1991	0.5494*	–

\*indicates  $P \leq 0.0001$ .  
doi:10.1371/journal.pone.0030927.t002

**Table 3.** Amino acid substitutions and resulting haplotypes in four studied genes from 51 parasite isolates.

Gene	Mutation	No. (%)	Haplotypes	No. (%)
Pfm <sup>1</sup> dr1 (86/184/1042)	N86 <b>Y</b>	1 (2.0)	NYN <sup>†</sup>	33 (64.7)
	Y184 <b>F</b>	14 (27.5)	NFN	13 (25.5)
	N1042 <b>D</b>	4 (7.8)	NYD	3 (5.9)
			YYN	1 (2.0)
			NFD	1 (2.0)
PfATP6 (89/226/438/465/710)	I89 <b>T</b>	5 (9.8)	IIAEG <sup>†</sup>	43 (84.3)
	I226 <b>V</b>	1 (2.0)	TIAEG	2 (3.9)
	A438 <b>D</b>	3 (5.9)	TIDEG	2 (3.9)
	N465 <b>S</b>	1 (2.0)	TIAKG	1 (2.0)
	E710 <b>K</b>	1 (2.0)	IIDEG	1 (2.0)
			IVAEG	1 (2.0)
		IIAKG	1 (2.0)	
Pfm <sup>6</sup> dr6* (R1/175/R2/353/R3/ SIN/823)	Y175 <b>S</b>	9 (17.7)	6 Y2L6+N <sup>†</sup>	0
	L353 <b>W</b>	1 (2.0)	8 Y2L6+-	25 (49.0)
	N823-	45 (88.2)	8 S2L6+-	5 (9.8)
			8 S2L6+N	4 (7.8)
			9 Y2L6+-	4 (7.8)
			8 Y2L5--	3 (5.9)
			6 Y2L6+-	2 (3.9)
			10Y2L6+-	2 (3.9)
			6 Y2W6+-	1 (2.0)
			7 Y2L6+-	1 (2.0)
			8 Y2L6+N	1 (2.0)
			8 Y3L6+-	1 (2.0)
		11Y2L5-N	1 (2.0)	
		12Y2L4+-	1 (2.0)	
Pfm <sup>1</sup> T (30/286/496)	I30 <b>N</b>	50 (98.0)	ISN <sup>†</sup>	1 (2.0)
	S286 <b>C</b>	1 (2.0)	NS-	48 (94.1)
	N496-	48 (94.1)	NSN	1 (2.0)
			NCN	1 (2.0)

<sup>†</sup>denotes the reference haplotype in 3D7; Letters in bold indicate mutated amino acids; - indicates deletion of the residue. No. (%) indicates the number and percentage of isolates harboring these mutations or haplotypes.

\*The mdr6 haplotypes: R1 repeats, number of N at positions 103–110; R2, number of NI at positions 267–270, R3, number of NIN at positions 717–734; the tri-peptide SIN at position 735–737 are shown as + (present) or - (deleted). doi:10.1371/journal.pone.0030927.t003

**Pfm<sup>6</sup>dr6.** Analysis of the coding region of the *pfm<sup>6</sup>dr6* gene from 51 parasite samples revealed mutations at residues 175 and 353 (Table 3). The Y175S mutation was found in 9 (17.7%) isolates, whereas L353W mutation was only found in one sample. In addition, this gene harbored two deletions. The tri-peptide SIN corresponding to positions 735–737 in 3D7 was deleted in four isolates, while the N residue at residue 823 was deleted in 45 (88.2%) isolates. Other polymorphisms all occurred in repeat sequences. For the microsatellite sequence corresponding to positions 103–110 in 3D7 (referred to as repeat R1), the number of the amino acid N ranged from 6 (in 3D7) to 12, with 8 as the

predominant one occurring in 39 (76.5%) isolates. 3D7 had two NI repeats at positions 267–270 (referred to as repeat R2). Whereas 50 (98.0%) isolates had two NI repeats, only one field isolate had three NI repeats. The third repeat sequence, referred to as repeat R3, contains NIN repeats at positions corresponding to 717–734 in 3D7. Whereas 46 (90.2%) isolates had 6 NIN repeats, 4 (7.8%) and 1 (2.0%) isolates had 5 and 4 NIN repeats, respectively. These polymorphisms gave rise to a total of 13 haplotypes (Table 3), but none of the field isolates had the same haplotype as 3D7. The most prevalent haplotype, which differs from 3D7 by 8 N residues in microsatellite repeats R1 and a codon 819 deletion, occurred in 25 (49.0%) parasite isolates.

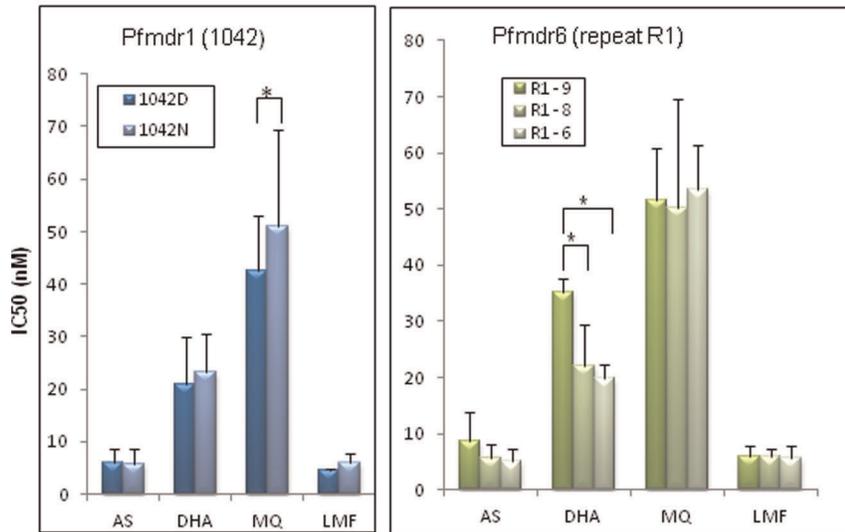
**PfMT.** Sequencing of the *pfm<sup>1</sup>T* gene detected a total of two amino acid substitutions at positions 30 and 286. The I30N mutation was found in all but one parasite isolate, whereas the S286C mutation was rare and found only in one isolate. In addition, the codon for amino acid N496 was deleted in the majority (94.1%) of the isolates. A total of four amino acid haplotypes were recognized with one (N30/286S/496-) accounting for 94.1% parasite isolates (Table 3). In comparison, all other haplotypes only occurred once in the samples.

### Correlation between Polymorphisms and *in vitro* Drug Sensitivities

Since the *pfcr* K76T mutation was fixed and the A220S mutation almost reached fixation (50/51), and no *pfm<sup>1</sup>dr1* amplification was detected in the studied parasite populations, associations could not be evaluated between these markers and drug responses. Thus, we analyzed the potential associations between point mutations in *pfm<sup>1</sup>dr1*, *pfATP6*, *pfm<sup>6</sup>dr6*, and *pfm<sup>1</sup>T* genes and drug responses. None of the mutations in the *pfm<sup>1</sup>dr1* and *pfATP6* genes had a significant association with altered responses to AS and DHA. Yet, the N1042D mutation in *pfm<sup>1</sup>dr1* was found to be associated with increased susceptibility to MQ (Figure 2, Table S3). SNPs in *pfm<sup>6</sup>dr6* and *pfm<sup>1</sup>T* genes that caused amino acid substitutions were rare. None of them was associated with significantly altered sensitivity to the four drugs tested ( $P > 0.05$ ). Neither did the deletion mutations (S735-/I736-/N737- and N823- in *pfm<sup>6</sup>dr6*, as well as N496- in *pfm<sup>1</sup>T*) affect the sensitivity to the four drugs. There are three simple amino acid repeats in *pfm<sup>6</sup>dr6*. Whereas no significant differences in drug sensitivity were detected between the parasites carrying different numbers of repeats in repeats R2 and R3, parasites carrying 9 repeats in repeat R1 were associated with significantly increased resistance to DHA ( $P < 0.05$ ) (Figure 2, Table S3). The difference between parasites carrying 8 and 9 R1 repeats remained significant after Bonferroni correction (Figure 2). In addition, 7 repeats in R1 were also significantly correlated with reduced sensitivity to LMF ( $P < 0.05$ ) (Table S3).

To determine whether certain haplotypes of *pfm<sup>1</sup>dr1*, *pfATP6*, *pfm<sup>6</sup>dr6*, and *pfm<sup>1</sup>T* genes are associated with altered drug responses, we compared the drug responses of the mutant haplotypes with those of the wild-type isolates. Comparison of *pfm<sup>1</sup>dr1* haplotypes with  $\geq 3$  (5.9%) prevalence showed that parasites carrying the *pfm<sup>1</sup>dr1* 1042D mutation showed increased sensitivity to MQ, whereas responses to other three drugs were not significantly different between the *pfm<sup>1</sup>dr1* haplotypes (Figure 3). All mutant haplotypes of *pfATP6* were below 4% in prevalence, and only 2 (3.9%) parasite isolates with the 89T/438D double mutations had significantly higher IC<sub>50</sub> values to AS (data not shown). For the *pfm<sup>6</sup>dr6* gene, we found a significant association for the haplotype carrying 9 N residues in microsatellite repeat R1 (9Y2L6+-) with significantly higher DHA IC<sub>50</sub> values when compared to both 3D7 and the haplotype 8Y2L6+-. These findings remained significant

Figure 2



**Figure 2. Association of SNPs and other polymorphisms in candidate genes with drug responses.** Only significant association of a SNP in *pfmdr1* and number variation of repeat R1 in *pfmdr6* with *in vitro* responses to artesunate (AS), dihydroartemisinin (DHA), mefloquine (MQ) and lumefantrine (LMF) are plotted here. IC<sub>50</sub> values are shown as mean + standard deviation. \*indicates significant difference ( $P < 0.05$ ) in sensitivity between the two alleles.

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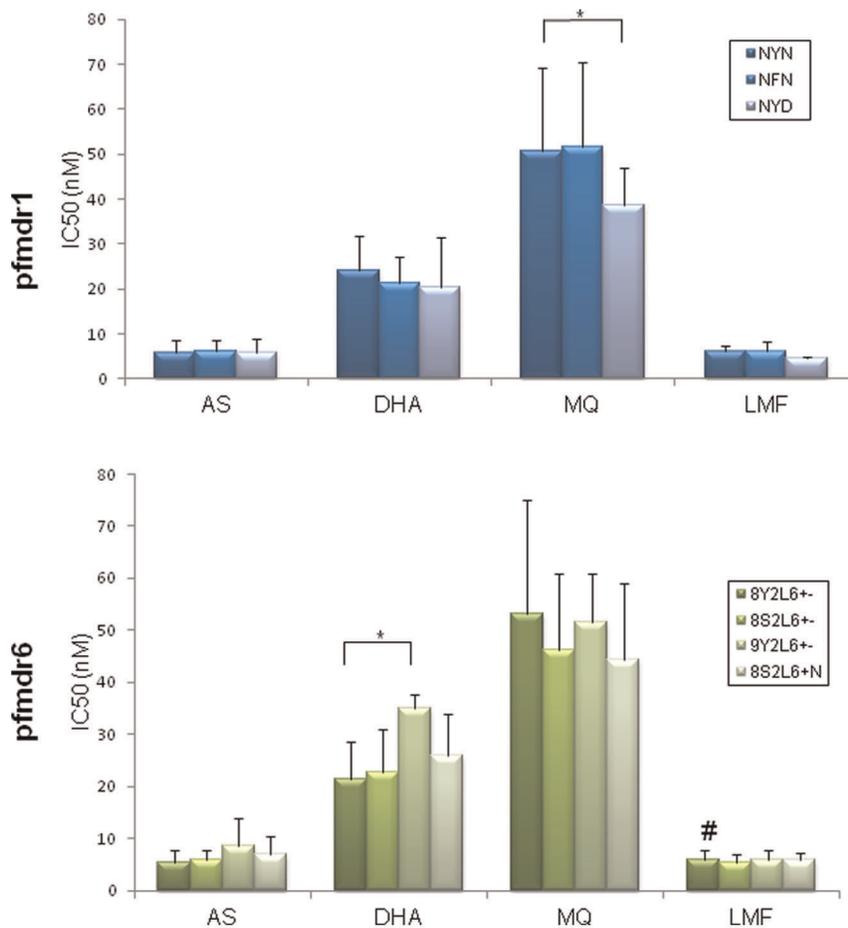
after Bonferroni correction (Figure 3). Furthermore, haplotype 8Y2L6+ showed significantly lower IC<sub>50</sub>s to LMF when compared to the wild-type 3D7. Interestingly, all of the haplotypes showed increased sensitivity to LMF when compared to 3D7. In comparison, none of the haplotypes in pMT were associated with altered drug sensitivity (data not shown).

## Discussion

The GMS in Southeast Asia has been a breeding ground of antimalarial drug resistance, where MDR parasites have emerged. Parasites in this region appear to have the ARMD (accelerated resistance to multiple drugs) genetic background, favoring development of resistance to new antimalarial drugs [58,59]. Further reflecting such a tendency, malaria parasites from this region have recently been found to exhibit reduced susceptibility or low-grade resistance to artemisinin derivatives [3,4]. This urgent situation calls for closer surveillance, containment and heightened research on resistance mechanisms. In some areas of the GMS such as the China-Myanmar border area, artemisinins have been used for over three decades, mostly as monotherapies prior to 2005. Earlier *in vitro* assays have already detected a trend of declining sensitivity to AS [46]. Surveillance at the Thai-Myanmar border also detected reduced sensitivity to artemisinins, MQ, and QN [60–62]. Therefore, understanding whether artemisinin-resistant parasites have spread to neighboring regions or emerged elsewhere in the GMS is essential for coordinating containment efforts. Here, we evaluated this situation at the China-Myanmar border area and assessed the *in vitro* sensitivity of recently collected parasite isolates to artemisinins and two amino alcoholic drugs. Our data showed that although these parasites were generally sensitive to AS, there were eight parasite isolates showing mean IC<sub>50</sub> values above 8 nM. In addition, *in vitro* sensitivity to DHA also had a more than 4-fold variation. Spearman's correlation test detected significant correlations in sensitivity between the two artemisinin derivatives, suggesting that these drugs have shared cellular

targets. Despite the fact that no careful clinical studies have been conducted in this region to rule out the lack of clinical resistance to artemisinins, our data suggest that reduced susceptibility to the artemisinins *might* have already emerged in some isolates (outliers) in the study parasite population. Therefore, close monitoring of resistance development at such sentinel sites is necessary. We have also included two aminoalcoholic drugs in the *in vitro* assays, since responses to MQ and LMF are often correlated with those to artemisinins [18,61]. At the China-Myanmar border area, MQ has never been deployed, but *in vitro* assays suggested the existence of reduced sensitivity to MQ with 37 parasite isolates showing higher mean IC<sub>50</sub> values than 3D7. Another perplexing finding is that the parasite isolates tested were highly sensitive to LMF, which is in sharp contrast to the parasites from Thailand that displayed more than 20-fold higher IC<sub>50</sub>s [63], although LMF has not been used in Thailand. The decreased sensitivity to LMF in Thailand could be related to the mass deployment of MQ in treating falciparum malaria, since sensitivities to MQ and LMF are correlated. Similarly, our samples collected in 2009 also showed significant correlation in IC<sub>50</sub> values between these two drugs. However, none of these two aminoalcoholic drugs showed positive correlation in IC<sub>50</sub> with the two artemisinin drugs. These are different from the *in vitro* drug studies carried out in other areas of the GMS [61,64–66]. The reason for such discrepancies was not clear, but may be related to the divergent antimalarial drug policies in these areas.

While the mode of action of artemisinins is not completely understood, several cellular targets have been proposed. Among them, PfATP6 has been suggested as a prime candidate target of artemisinins [29,30], and studies with field parasite isolates from French Guiana seemed to support this suggestion [31]. However, since these reports, results from subsequent studies appear to contradict the hypothesis of PfATP6 as a specific target of artemisinins. First, artemisinin treatment of *P. falciparum* did not alter the morphology of endoplasmic reticulum, where PfATP6 is supposed to be localized [67]. Second, purified recombinant



**Figure 3. Association of haplotypes in candidate genes with drug responses.** Only association of major haplotypes in *pfm1* and *pfm6* with *in vitro* responses to artesunate (AS), dihydroartemisinin (DHA), mefloquine (MQ) and lumefantrine (LMF) are plotted. IC<sub>50</sub> values are shown as mean + standard deviation. \* indicates significant difference ( $P < 0.05$ ) in sensitivity between the haplotypes after Bonferroni correction. # indicates that this *pfm6* haplotype was significantly more sensitive to LMF than 3D7. doi:10.1371/journal.pone.0030927.g003

PfATP6 did not seem to bind artemisinins, nor was it inhibited by artemisinins [68,69]. Third, allelic exchange experiments aiming to determine whether the PfATP6 L283E mutation is linked to a drastic reduction of artemisinin sensitivity only provided tangential evidence supporting this suggestion [70]. In addition, our recent allelic exchange experiment did not detect differences in sensitivity to artemisinin derivatives between parasites carrying either S769 or 769N [71]. Further, an African parasite isolate carrying the S769N mutation did not show increased resistance to artemisinins [34]. In the Thai-Cambodian border region, where resistance to artemisinins has recently emerged, these two mutations have not been detected either [42]. We and others further confirmed the lack of these mutations in parasites from the China-Myanmar border area, where artemisinins have been deployed extensively for over 30 years [38]. In fact, most parasites from our study carried the wild-type *PfATP6* gene, and no significant association could be established between the rare mutations in *pfATP6* and artemisinin resistance. Collectively, these data potentially suggest that PfATP6 might not play a major and direct role in artemisinin resistance.

The malaria parasite encodes many transporters and some of them such as *pfert*, *pfmdr1* and *pfmrp* have been strongly connected with antimalarial drug resistance [72–74]. Parasite resistance to multiple drugs has been associated with SNPs and increased copy

number of the ABC transporter family member *pfmdr1*. *Pfmdr1* amplification is correlated with resistance to MQ, QN, HF, and artemisinins [20,75]. Laboratory experiments confirmed that an increase of *pfmdr1* copy number can be induced by stepwise MQ selection [76], whereas knockdown of *pfmdr1* expression results in increased susceptibility to MQ, QN, and artemisinin [77]. Moreover, recent laboratory selection studies strongly implicated *pfmdr1* gene amplification as a major determinant of induced artemisinin resistance [78,79]. *Pfmdr1* also harbors many point mutations: the 86Y mutation which modifies resistance to CQ is associated with increased sensitivity to MQ and artemisinin [16], whereas mutations at the C-terminal end, which are prevalent in South America, contribute to QN resistance but enhance MQ and artemisinin sensitivity [18]. Consistently, administration of artemether-LMF has selected for increased frequency of the N86 allele [32,80]. In Thailand and Cambodia, *pfmdr1* gene amplification has become increasingly prevalent in field parasite populations and was responsible for resistance to MQ and declining efficacy of ACTs [19,22,23,26,28]. In this case, *pfmdr1* amplification has been strongly tied to the extensive deployment of MQ in this region. At the China-Myanmar border, however, *pfmdr1* gene amplification has not been observed, consistent with the fact that MQ has never been used in this region. Overall, the frequencies of *pfmdr1* N86Y and C-terminal mutations which confer increased sensitivity to

artemisinins were very low in the GMS [61,64]. Our results from the China-Myanmar border further verified these findings [81]. Yet, despite the implied significance of *pfmdr1* in artemisinin resistance, in our study, *pfmdr1* was not associated with the reduced artemisinin susceptibility recently unveiled in western Cambodia [42]. The uncommon *pfmdr1* polymorphisms from these parasites offer opportunities for investigations of mechanisms other than *pfmdr1*.

The clear link between polymorphisms of transporter genes and antimalarial drug resistance has prompted us to evaluate other genes encoding putative transporters. We have chosen *pfmdr6*, another ABC transporter family member, and *pfMT*, a putative metabolite/drug transporter, since these two genes have been shown to be under recent positive selection from a genome-wide association study [43]. Both genes were found to harbor small numbers of nonsynonymous SNPs, whereas *pfmdr6* also have polymorphisms in three simple repeats. Compared with 3D7, the Y175S mutation and the 823 codon deletion had reached 17.7 and 88.2% of prevalence, respectively. However, none of the point mutations were associated with altered drug sensitivity. Yet, the microsatellite repeat R1, which has seven different alleles (ranging from 6 to 12 repeats), appeared to affect the *in vitro* sensitivity to DHA. Specifically, 9 Ns in R1 was associated with reduced sensitivity to DHA. This is reminiscent of the effect of the microsatellite repeats in the *pfNHE1* gene on *in vitro* sensitivity to QN [50,82]. Of the 13 haplotypes detected within this gene, 8 were rare haplotypes, each being represented by one or two isolates. Again, the haplotype with 9 Ns in R1 was associated with significantly higher IC<sub>50</sub> value to DHA than others. Compared to 3D7, two mutations in *pfMT* (I30N and N496-) were predominant in the samples analyzed, which together gave rise to a predominant haplotype. Correlation studies did not detect any association of the mutations or haplotypes with significant altered drug sensitivity. However, we would like to exercise caution in this finding since previous *in vitro* studies of parasite resistance to AS have shown poor correlations with clinical *in vivo* studies. Furthermore, significant associations between these rare haplotypes and drug responses were at most based on a small number of parasite isolates carrying such mutations. Therefore, such an association may not hold if more samples carrying these mutations were analyzed.

Despite extensive efforts aimed at elucidating the mechanism of artemisinin resistance in *P. falciparum* using the candidate gene approach, most of the mutations encountered in candidate genes did not account for reduced sensitivity to artemisinins in parasites from clinical studies in western Cambodia [42]. Our *in vitro* studies using parasites from the China-Myanmar border area seem to convey a similar conclusion. However, analysis of parasites originating from the Thai-Myanmar border region implied that *pfmdr1* copy number and a new point mutation (F1226Y) are significantly associated with *in vitro* response to artemisinins [65].

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This discrepancy may reflect the intrinsic difference between *in vitro* assay and *in vivo* clinical studies. In addition, it may also be due to divergent genetic backgrounds of the parasite populations, since the significance of *pfmdr1* point mutations for CQ and QN resistance appears to depend on the genetic background of the parasites [17,18]. Meanwhile, another very relevant finding of this study is the existence of dramatic differences in drug responses and genotypes between our studied parasite population and other populations in the GMS. As malaria control is being intensified, geographical separation of parasite populations will occur. Differences in regional drug policy will inevitably exert distinctive selection pressures on parasites. Therefore, investigations into drug resistance mechanisms using different populations may help define the bona fide genetic determinants of artemisinin resistance. Furthermore, since artemisinin resistance appears to be a multi-genetic trait, mutations in multiple genes may have cumulative effects on parasite's response to artemisinins. Consequently, genetic analysis at multiple loci and association studies with combined haplotypes may generate meaningful insights into the mechanisms of artemisinin resistance [65]. Genome-wide approaches such as SNP array and deep sequencing technologies may be crucial in identifying the molecular basis of the artemisinin resistance. It is also imperative that close resistance surveillance be continued at sentinel sites where artemisinins have been deployed extensively in the past so that the emergence and spread of artemisinin resistance is carefully monitored.

## Supporting Information

**Figure S1 Value of the *pfmdr1* copy number in 51 *P. falciparum* isolates.** Each symbol indicates the *pfmdr1* copy number in one isolate. (TIF)

**Table S1 Primers used in this study.** (DOC)

**Table S2 *In vitro* IC<sub>50s</sub> (nM) of cultured field isolates to four antimalarial drugs** (mean ± standard deviation). (DOC)

**Table S3 *In vitro* IC<sub>50s</sub> (nM) of parasite isolates to artesunate (AS), dihydroartemisinin (DHA), mefloquine (MQ) and lumefantrine (LMF) stratified by mutations in *pfmdr1*, *pfATP6*, *pfmdr6* and *pfMT*.** (DOC)

## Author Contributions

Conceived and designed the experiments: LC ZY. Performed the experiments: HM DP JL ZZ ZW. Analyzed the data: HM DP QF HW LC ZY. Contributed reagents/materials/analysis tools: LW RZ. Wrote the paper: DP LC ZY.

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